

Advanced Multiplex PCR Assay for Differentiation of *Brucella* Species[▽]

Sung-Il Kang, Moon Her,* Jong Wan Kim, Ji-Yeon Kim, Kyung Yuk Ko,
Yun-Mi Ha, and Suk Chan Jung

OIE Reference Laboratory for Brucellosis, Bacteriology Division, National Veterinary Research and
Quarantine Service (NVRQS), Anyang, Gyeonggi-do 430-757, South Korea

Received 14 March 2011/Accepted 31 May 2011

Two new primer sets of a 766- and a 344-bp fragment were introduced into the conventional Bruce-ladder PCR assay. This novel multiplex PCR assay rapidly and concisely discriminates *Brucella canis* and *Brucella microti* from *Brucella suis* strains and also may differentiate all of the 10 *Brucella* species.

The alphaproteobacterial genus *Brucella* consists of 10 species: *B. abortus*, *B. canis*, *B. suis*, *B. ovis*, *B. neotomae*, *B. melitensis*, *B. ceti*, *B. pinnipedialis*, *B. microti*, and *B. inopinata* (3, 5, 16, 17). *Brucella* species show a host preference, but some strains can be transmitted among a variety of animals, including humans (11, 12, 14, 18).

To accelerate effective prevention and control of brucellosis, a fast and accurate identification method is necessary. Many studies have developed PCR-based assays for the discrimination of *Brucella* species (2, 4, 7, 8, 10). Recently, López-Goni et al. reported that the Bruce-ladder PCR assay could differentiate most *Brucella*

species, including marine mammal and vaccine strains *B. abortus* S19, *B. abortus* RB51, and *B. melitensis* Rev.1 (6, 10, 13). However, these assays did not solve the problem of erroneous identification of *B. canis* isolates (such as *B. suis*) and basic differentiation between two marine mammal *Brucella* species (*B. ceti* and *B. pinnipedialis*) (9, 13, 15). Therefore, the aim of this study was to develop a fast, simple, and accurate one-step multiplex PCR assay to differentiate 10 *Brucella* species using 22 reference strains and 106 field isolates in South Korea.

To discriminate between *B. canis* and *B. suis*, alignments of their whole-genome sequence or each biovar short-gun

Strains	Nucleotide sequence (5' → 3')						Remarks
	1	19	105	270		339	
<i>B. melitensis</i> bv1	<u>CCAACCGTATGTCCTCTCT</u>GCCCT.....AAGAAGGCTGGAAGAAGACAGATCAGAAAAGSCCAAGTGTGAGCAATAGGCTTTCGAACGTAGCCTGCGGT						BME II 0722
<i>B. abortus</i> bv1						94bp deletion
<i>B. canis</i>						12bp deletion
<i>B. suis</i> bv1-5						
<i>B. ovis</i>						
<i>B. neotomae</i>T.....						point mutation, 1bp deletion
<i>B. pinnipedialis</i>						
<i>B. ceti</i>TCCG.....CAATATCC.....C.....AGC.....AAC.....GC.....CTGGCGCACCCTGC.....CCC.....CAACA.....CCTGATATACCACCG						139bp replacement
<i>B. microti</i>						
<i>B. canis</i> HN-3						12bp deletion
<i>B. canis</i> JB-1						12bp deletion
<i>B. canis</i> HSY-6						12bp deletion
Strains	Nucleotide sequence (5' → 3')						Remarks
	340	363	371	409	559	745	766
<i>B. melitensis</i> bv1	GGTATATCAGGCGTGTTCGCGGTG-----CGCCAGTTGCGGTTTGTTCGCGGATATTGCTCGGACTA.....CCCCG.....CGTCGAACACCAAGTTCCCGCA						BME II 0721
<i>B. abortus</i> bv1						
<i>B. canis</i>GCGGGTG.....						7bp insertion
<i>B. suis</i> bv1-5GCGGGTG.....						7bp insertion
<i>B. ovis</i>GCGGGTG.....						7bp insertion
<i>B. neotomae</i>GCGGGTG.....						7bp insertion
<i>B. pinnipedialis</i>GCGGGTG.....						7bp insertion
<i>B. ceti</i>	CAGGCTA.GTT.AAAGC.TAT.CTCACGCTTGCC.TTCTGA.CTG.TCTTCCAGC.TCTT.T.....						972bp deletion
<i>B. microti</i>GCGGGTG.....						7bp insertion
<i>B. canis</i> HN-3GCGGGTG.....						7bp insertion
<i>B. canis</i> JB-1GCGGGTG.....						7bp insertion
<i>B. canis</i> HSY-6GCGGGTG.....						7bp insertion

FIG. 1. Alignment for specific gene fragments (766 bp) of representative *B. canis* isolates and *Brucella* reference strains. The *B. canis* isolates are indicated as HN-3, JB-1, and HSY-6. The primer set is underlined. Dots indicate consensus sequence; dashes indicate deletions.

* Corresponding author. Mailing address: OIE Reference Laboratory for Brucellosis, Department of Bacteriology, National Veterinary Research and Quarantine Service (NVRQS), Anyang, Gyeonggi-do 430-757, South Korea. Phone: 82 31 467 1776. Fax: 82 31 467 1778. E-mail: herm@korea.kr.

[▽] Published ahead of print on 10 June 2011.

Strains	Nucleotide sequence (5' → 3')				Remarks
	1	88	239	257	
<i>B. melitensis</i> bv1	CTACTCAAGGACTTCTGCTATTCTACGTGTACGGGACTATCACCCACTTCGGTCGAGTTTCCAACTCGTTCCACTTTATTACAAAAA.....GCTGTGCTGTTCGCTCA				BME1 r02
<i>B. abortus</i> bv1				
<i>B. canis</i>				
<i>B. suis</i> bv1				
<i>B. ovis</i>				
<i>B. neotomae</i>				
<i>B. ceti</i>				
<i>B. pinnipedialis</i>				
<i>B. inopinata</i>C·A-----				14bp deletion
<i>B. microti</i>	<u>CTACTCAAGGACAACAGGTG</u>G.....CAA·CC.....GG·TC.....C·TATT.....C·A-----				14bp deletion

Strains	Nucleotide sequence (5' → 3')				Remarks
	258			369	
<i>B. melitensis</i> bv1	CGCGCCGTACGCATTCCAGAGGAATGCTGGCGCTCCGCGGGGGCGGCCACATGGGCCGACGACCTAGCGGTCTGTATGGGTGTAAACCATCCAGCGTGTCTCAACAAA				BME1 r02
<i>B. abortus</i> bv1				1bp deletion
<i>B. canis</i>				1bp deletion
<i>B. suis</i> bv1				1bp deletion
<i>B. ovis</i>				1bp deletion
<i>B. neotomae</i>				1bp deletion
<i>B. ceti</i>				1bp deletion
<i>B. pinnipedialis</i>				1bp deletion
<i>B. inopinata</i>	T.....-CT·A·AAGGCAG·AAGT·AGTAG·CAATAA·G·A·T··TTT---TT·A·CT·CC·ACTGCC·A·TGCCCTATT--GCCTTAAAC-GACACA				109bp replacement
<i>B. microti</i>	T.....-CT·A·AAGGCAG·AAGT·AGTAG·CAATAA·G·A·T··TTT---TT·A·CT·CC·ACTGCC·A·TGCCCTATT--GCCTTAAAC-GACACA				109bp replacement

FIG. 2. Alignment of specific gene fragment (344 bp) of *B. microti* compared to other *Brucella* species. The primer set is underlined. Dots indicate consensus sequence; dashes indicate deletions.

sequence were performed extensively using CLC Main Workbench (version 5.7) software (Insilicogen Inc., South Korea). Compared to five biovars of *B. suis*, a specific 12-bp deleted genetic site was discovered in the BME2 0722 genetic region of *B. canis* (Fig. 1). Other *Brucella* strains, except for *B. abortus*, did not show this gene deletion. However, in *B. abortus*, a 94-bp gene deletion that partially consisted of the 12-bp deleted site was detected. The reverse primer site confirmed an extensive 972-bp gene deletion site at BME2 0721 in *B. ceti*. This deletion site was not found in other *Brucella* species such as *B. pinnipedialis*. Based on the results from the alignment, a new primer set of 766 bp was designed to replace the 794-bp fragment of the BME2 436f-435r primer set in the Bruce-ladder PCR assay (10). This new primer set consisted of 5'-CCAACCGTATGTCCTCTCT-3' (forward) and 5'-TGCGGGAAGTGGTGTTCGACG-3' (reverse). Another primer set designed from the BME1 r02 region, which was aligned with 23S rRNA sequences from other *Brucella* species, was also included for the detection of *B. microti* (Fig. 2). The forward primer was designed by a site genetically mutated compared to other *Brucella* species, and the reverse primer was designed at a 109-bp replacement genetic site of the BME1 r02 region. A distinctive sequence site of 344 bp in *B. microti* was recognized using the primer set 5'-CTACTCAAGGACAACAGGTG-3' (forward) and 5'-TGTGTCGTTTAAGGCAATAGG-3' (reverse). Seven primer sets, except for BME2 436f-435r primer, were synthesized in accordance with the Bruce-ladder assay as described previously (10). PCR amplification was performed using 2× PCR premix kit (Cosmo Genetech, South Korea). PCR conditions consisted of an initial denaturing at 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 60°C for 2 min, and 72°C for 1 min, and a final extension at 72°C for 5 min.

As a result, the PCR using the new primer set of 766 bp distinctly discriminated *B. canis* and *B. suis* strains (Fig. 3A). In

a multiplex PCR assay including this primer set, *B. melitensis* (3 biovars), *B. suis* (5 biovars), *B. ovis*, *B. neotomae*, *B. pinnipedialis*, and *B. microti* were amplified selectively, whereas *B. abortus* (biovars 1 to 6 and 9, RB51, and S19), *B. canis*, *B. ceti*, and *B. inopinata* reference strains did not generate any amplicons (Fig. 3B). Also, 76 *B. canis* strains used in this study unambiguously showed as not being amplified by this specific primer set (data not shown). Therefore, the primer set of the 794-bp fragment in the Bruce-ladder PCR assay was replaced by our novel primer set of 766 bp, which can distinguish *B. canis* from *B. suis* strains and could discriminate between *B. ceti* and *B. pinnipedialis* reference strains (Fig. 3b). This novel primer might be used to differentiate between *B. ceti* and *B. pinnipedialis* even though it was not applied to marine mammal *Brucella* isolates.

Audic et al. analyzed the whole-genome sequence of *B. microti* and reported that the most noticeable difference between *B. microti* and other *Brucella* species was in the 23S rRNA gene (1). Based on this report, a new designed primer set of a 344-bp fragment specifically amplified only *B. microti*; however, it did not detect other *Brucella* species and 106 field isolates used this study (Fig. 3B). Furthermore, the recent *B. inopinata* strain was distinguished from other *Brucella* species because of not being amplified by the two new primers as it was by the previously described primer sets (1,071 bp) (13).

Consequently, the novel multiplex PCR assay was a rapid and robust diagnostic method to discriminate *B. canis* and *B. microti* from other *Brucella* species, including *B. suis*, in a single step. Moreover, this assay discriminated all 10 *Brucella* species, including marine mammalian reference strains. This multiplex PCR assay could be readily utilized as a genetic screening tool for *Brucella* strains isolated from animals and humans, not only for the prevention and control of the disease but also in any microbiology laboratory worldwide. In addition, this assay could contribute to efforts to eradicate brucellosis in underde-

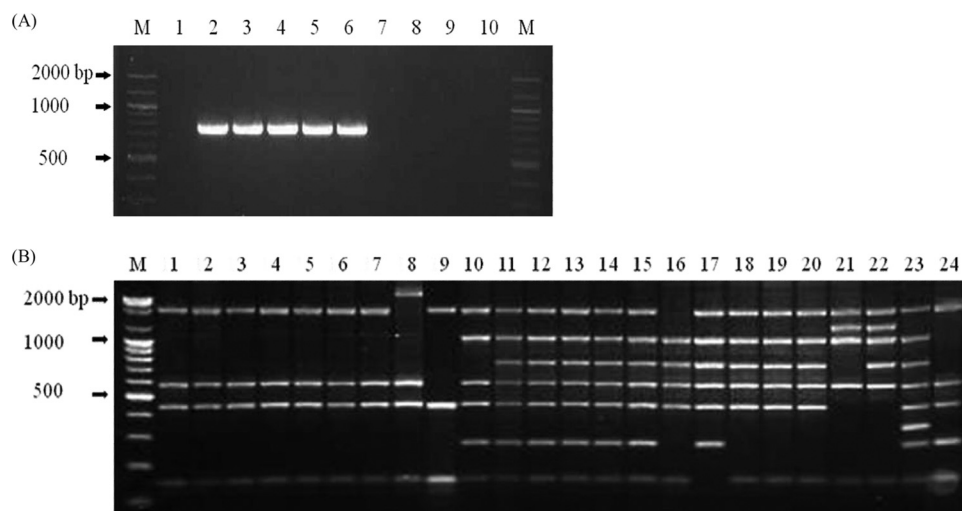


FIG. 3. (A) PCR products of *B. canis* and *B. suis* strains amplified using the novel primer set of 766 bp. Lanes M, 100-bp DNA ladder; lane 1, *B. canis* ATCC 23365; lanes 2 to 6, *B. suis* biovars 1 to 5, respectively; lanes 7 to 10, *B. canis* isolates. (B) PCR products from *Brucella* species amplified using the advanced multiplex PCR assay. Lane M, 100-bp DNA ladder; lanes 1 to 7, *B. abortus* biovars 1 to 6 and 9, respectively; lane 8, *B. abortus* RB51; lane 9, *B. abortus* S19; lane 10, *B. canis* ATCC 23365; lanes 11 to 15, *B. suis* biovars 1 to 5; lane 16, *B. ovis*; lane 17, *B. neotomae*; lanes 18 to 20, *B. melitensis* biovars 1 to 3; lane 21, *B. ceti*; lane 22, *B. pinnipedialis*; lane 23, *B. microti*; lane 24, *B. inopinata*.

veloped or developing countries with limited financial resources.

This study was supported by a fund of the Veterinary Science Technical Development project of the National Veterinary Research and Quarantine Service, South Korea (project no. C-AD13-2010-12-01).

REFERENCES

- Audic, S., M. Lescot, J. M. Claverie, and H. C. Scholz. 2009. *Brucella microti*: the genome sequence of an emerging pathogen. *BMC Genomics* **10**:352.
- Bricker, B. J., and S. M. Halling. 1994. Differentiation of *Brucella abortus* bv. 1, 2, and 4, *Brucella melitensis*, *Brucella ovis*, and *Brucella suis* bv. 1 by PCR. *J. Clin. Microbiol.* **32**:2660–2666.
- Corbel, M. J. 1997. Brucellosis: an overview. *Emerg. Infect. Dis.* **3**:213–221.
- Ewalt, D. R., and B. J. Bricker. 2000. Validation of the abbreviated *Brucella* AMOS PCR as a rapid screening method for differentiation of *Brucella abortus* field strain isolates and the vaccine strains, 19 and RB51. *J. Clin. Microbiol.* **38**:3085–3086.
- Foster, G., B. S. Osterman, J. Godfroid, I. Jacques, and A. Cloeckaert. 2007. *Brucella ceti* sp. nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred hosts. *Int. J. Syst. Evol. Microbiol.* **57**:2688–2693.
- García-Yoldi, D., et al. 2006. Multiplex PCR assay for the identification and differentiation of all *Brucella* species and the vaccine strains *Brucella abortus* S19 and RB51. *Clin. Chem.* **52**:779–781.
- Gopaul, K. K., M. S. Koylass, C. J. Smith, and A. M. Whatmore. 2008. Rapid identification of *Brucella* isolates to the species level by real time PCR based single nucleotide polymorphism (SNP) analysis. *BMC Microbiol.* **8**:86.
- Herman, L., and H. De Ridder. 1992. Identification of *Brucella* spp. by using the PCR. *Appl. Environ. Microbiol.* **58**:2099–2101.
- Koylass, M. S., et al. 2010. Comparative performance of SNP typing and 'Bruce-ladder' in the discrimination of *Brucella suis* and *Brucella canis*. *Vet. Microbiol.* **19**:450–454.
- López-Góñi, I., et al. 2008. Evaluation of a multiplex PCR assay (Bruce-ladder) for molecular typing of all *Brucella* species, including the vaccine strains. *J. Clin. Microbiol.* **46**:3484–3487.
- Lucero, N. E., et al. 2005. Unusual clinical presentation of brucellosis caused by *Brucella canis*. *J. Med. Microbiol.* **54**:505–508.
- Maquart, M., M. S. Zygmunt, and A. Cloeckaert. 2009. Marine mammal *Brucella* isolates with different genomic characteristics display a differential response when infecting human macrophages in culture. *Microbes Infect.* **11**:361–366.
- Mayer-Scholl, A., A. Draeger, C. Göllner, H. C. Scholz, and K. Nöckler. 2010. Advancement of a multiplex PCR for the differentiation of all currently described *Brucella* species. *J. Microbiol. Methods* **80**:112–114.
- Moreno, E., A. Cloeckaert, and I. Moriyón. 2002. *Brucella* evolution and taxonomy. *Vet. Microbiol.* **90**:209–227.
- Office International des Épidémiologies. 2008. Manual of diagnostic tests and vaccines for terrestrial animals, 6th ed. Office International des Épidémiologies, Paris, France.
- Scholz, H. C., et al. 2010. *Brucella inopinata* sp. nov., isolated from a breast implant infection. *Int. J. Syst. Evol. Microbiol.* **60**:801–808.
- Scholz, H. C., et al. 2008. *Brucella microti* sp. nov., isolated from the common vole *Microtus arvalis*. *Int. J. Syst. Evol. Microbiol.* **58**:375–382.
- Seleem, M. N., S. M. Boyle, and N. Sriranganathan. 2010. Brucellosis: a re-emerging zoonosis. *Vet. Microbiol.* **140**:392–398.